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WASHINGTON DC 20523

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MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18  
FROM: AID/SCI, Victoria Ose *VO*  
SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 7. 272  
PR #1

Attachment

*1 copy*

PN-118A570  
7. 272

PROGRESS REPORT NO. 1

EARLY DETECTION OF LYMPHATIC FILARIASIS AND ATTEMPT AT DEFINING  
PROTECTIVE ANTIGENS

A RESEARCH PROJECT  
USAID/PSTC PROGRAM  
Grant No. 936-5542-G-00-7030-00

Submitted by

WANLOP CHUSATTAYANOND, PH.D.

PROJECT LEADER

DEPARTMENT OF MICROBIOLOGY  
FACULTY OF SCIENCE, MAHIDOL UNIVERSITY

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## PROJECT PROFILE

Country: Thailand

Grant No.: 936-5542-G-00-7030-00

Program: Program on Science and Technology Cooperation

Project Title: Early Detection of Lymphatic Filariasis and Attempt at Defining Protective Antigens

Project Leader: Dr. Wanlop Chusattayanond

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Authorized Officer: Dr. Pairote Prempree  
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Mahidol University

Total Project Budget: US \$149,930

Project Duration: 3 years

Reporting Period: August 1987 - January 1988

Budget Allocation  
for This Period: US \$40,946.94

## INTRODUCTION AND OBJECTIVES

Lymphatic filariasis is a serious disease effecting approximately 90 million people in the tropics with a population at risk of more than 900 millions. It is mosquito-transmitted disease which is caused by Wuchereria bancrofti, Brugia malayi and B. timori. While W. bancrofti is found throughout the wet tropical world, B. malayi is confined to Asia and B. timori is only found on the islands of Timor, Flores, Alor and Roti of Indonesia (Wheeling *et al*, 1975).

In Thailand, W. bancrofti is found in the Western part of the country along the border with Burma while the endemic area of B. malayi is in the South. It has been reported that the control programme of filariasis in Southern Thailand during 1961-1979 which covered 6 endemic areas of malayan filariasis with a total population of about 1.5 million (people at risk) was successful. However, the Filariasis Division, Ministry of Public Health recently (1982-1985) reported that new areas which had been previously inaccessible to governmental personnels were found to be highly endemic with infection rates of 1.16% to 4.6%. As is often the case epidemiological data reflects the extent and limitation of the survey carried out and usually represent underestimation of the problems. In Thailand and most developing countries, this underestimation is found all the time due to under-reporting and relative limitation of personnels and inavailability of proper diagnostic tools for field surveys. Diagnostic test which have been currently used is the detection of microfilaraemia which occur at night. Therefore, development of a simple, sensitive, specific, accurate and affordable diagnostic systems will make case detection much simpler. Furthermore, a diagnostic system which can detect antigens of early human stages of the parasite in asymptomatic subjects in endemic areas will not only make

chemotherapy more effective but will also help to interrupt the life cycle of the parasites. By treating the subjects before they become microfilaraemic, transmission of the parasites will be reduced.

The overall aim of this research is to improve disease control by developing better immunodiagnostic tests for lymphatic filariasis as well as new tests for the detection of early, i.e., pre-microfilaraemic stage of filarial infection based on detection of stage specific antigens in the serum and urine.

The specific objectives are to

1. analyse antigenic structure for the development of immunological reagents specific for B. malayi and W. bancrofti and those specific for various stages of the parasites.

2. devise simple, sensitive, specific, accurate and inexpensive immunoassays for the diagnosis of clinical lymphatic filaria as well as for the detection of early asymptomatic infection.

## MATERIALS AND METHODS

### Experimental animals

Cats and jirds were used as definitive hosts of Brugia pahangi and B. malayi. These existing experimental animal models have been established with a re-entry grant from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (WHO/TDR) to the Principal Investigator.

Cats less than one year old were obtained from houses in central Bangkok that possessed unwanted kittens. They were examined for natural infection of filarial parasites before using for experimental infection. Rabies vaccine and anthelmintic drug were given a few weeks after arriving the Animal Centre, Faculty of Science, Mahidol University. These cats were fed twice a day with appropriately cooked fishes mixed with steamed rice. Commercial cat biscuits were also given for supplementation. Jirds, on the other hand were the offsprings of those kindly given by Dr. D.A. Denham of London School of Hygiene and Tropical Medicine, London, England and Dr. Mak Joon Wah of Institute for Medical Research, Kuala Lumpur, Malaysia. Water and Commercial pellet diet were given ad libitum.

Rabbits were used for the production of hyperimmune sera against filarial and non-filarial parasite antigens. The animals were obtained from the Animal Centre, Faculty of Science, Mahidol University. They were fed ad libitum with water and commercial pellet diet throughout the experimental period.

### Brugia pahangi and B. malayi

The existing experimental animal models consist of susceptible mosquitoes (Aedes aegypti, carrying homozygous fm genes); cats and jirds which harbour full life cycles of B. pahangi and B. malayi. Various stages of the parasites were obtained by the following techniques.

### Infective third-stage larvae

The mosquitoes, Aedes aegypti were allowed to feed on microfilaraemic cats which have been anaesthetized by intraperitoneal injection with sodium pentobarbitone (Sagatal-May and Baker, Ltd.). The cats were previously infected subcutaneously with 70-90 infective third-stage larvae and only those harbouring 3-4 microfilariae per  $\mu$ l of peripheral blood (counted in chambers by the techniques of Denham et al, 1971) were used. Ten to twelve days after feeding, the mosquitoes were killed and lightly crushed with a glass test tube in a small volume of phosphate buffered saline (PBS) pH 7.2-7.4 on a glass plate and washed into the sieve (mesh size 75  $\mu$ m) of a Baermann's apparatus filled with PBS. Third-stage larvae migrated rapidly through the mesh of the sieve and were collected from the bottom of the apparatus (Denham et al, 1984, Chusattayanond and Denham, 1986). Four to five layers of gauze were placed on top of the sieve when clean larval preparations were needed.

### Adult worms

Adult Brugia pahangi and B. malayi were obtained from peritoneal cavity of jirds previously infected with 100-200 infective third-stage larvae of the parasite for more than 8 weeks (McCall et al, 1973).

### Microfilariae

Microfilariae were obtained in peritoneal washing fluid of infected jirds. Phosphate buffered saline was used and the parasite was separated from host cells by density centrifugation on Percoll (density 1.13 g/ml, Pharmacia) in 0.25 M sucrose (Chandrashekar, 1984). The larvae were also obtained in vitro when adult female worms were maintained in BME (Basal Medium Eagle, BRL) at 37°C in the presence of 5% CO<sub>2</sub>.

## Other filarial worms

### *Dirofilaria repens*

A microfilaraemic cat was found in Southern Thailand and brought to the Faculty of Science, Mahidol University by the Filariasis Division, Department of Communicable Diseases Control, Ministry of Public Health, Bangkok one month ago. The infection is still active and the animal has been taken care by the Animal Centre of the Faculty.

### *Dirofilaria immitis*

Adult male and female dog heart worms were obtained from microfilaraemic stray dogs sacrificed by the Bangkok Munciple Dog Pound. The worms were maintained in RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum at 37°C in the presence of 5% CO<sub>2</sub> for 2 weeks. Microfilariae were harvested daily from the medium after centrifugation at 1,800 rpm for 10 min.

## Non-filarial parasites

### *Angiostrongylus cantonensis* infective larvae

A complete life cycle of *Angiostrongylus cantonensis* has been established in the Department of Microbiology, Faculty of Science, Mahidol University (Techasoponmani and Sirisinha, 1980, Dharmkrong-at and Sirisinha, 1983). To obtain the infective larvae, fresh water snails, *Australorbis glabratus* were infected with first-stage larvae which had been separated from rat faeces. Three weeks after infection, infective third-stage larvae were freed from the snail tissues by artificial digestion with 1% pepsin, pH 2.0. The larvae were separated from the fluid by gravitational sedimentation.

### Gnathostoma spinigerum infective larvae

The infective third-stage larvae of this parasite were collected from the liver of naturally infected eels bought from a local market in Bangkok. Liver from several eels were pooled and digested at 37°C overnight with pH 2.0 pepsin solution (Eharmkrongrat et al, 1986). Living larvae were identified using morphological criteria described by Daengsvang (1980). The larvae were individually picked and separated from the digested liver tissue.

### Toxocara canis

Adult male and female worms were collected from feces of a puppy after receiving anthelmintic drug. The worms were washed several times with PBS before keeping frozen at -20°C in a small amount of saline.

### Parasite antigens

Three different types of antigens were prepared from these parasites. They can be classified as somatic antigens, excretory-secretory antigens (ES) and moulting antigens.

#### Somatic antigens

Worms of all stages of the parasites collected for somatic antigen preparations were washed 3-4 times with PBS and kept frozen at -20°C in a small amount of saline until adequate amount was obtained. When preparing the antigens, frozen worms were thawed and homogenized in a ground glass tissue grinder in the presence of protease inhibitors (0.1 mM TPCK, L-1-tosylamide-2-phenyl-ethylchloromethyl ketone and 0.1 mM PMSF, Phenyl methylsulfonyl fluoride, SIGMA). The homogenate was broken up further by brief sonication. The sonicate was ready to be used as a whole worm extract (WWE) or was centrifuged at 10,000 rpm for 30 min and the supernatant was obtained as an aqueous extract (AE). All the

processes described were performed at 4°C. The antigens were stored at -20°C until use.

#### Excretory-secretory and moulting antigens

Infective third-stage larvae of *B. pahangi* were cultured to the fourth-stage by the method of Mak et al. (1983). The infected mosquitoes were killed and lightly crushed in a small volume RPMI 1640 (with L-glutamine, Gibco, USA) buffered with sodium bicarbonate and HEPES to pH 7.2-7.4 supplemented with 200 U/ml of penicillin and 200 µg/ml of streptomycin. The larvae were prepared by the technique described above using a Baermann's apparatus filled with the RPMI 1640 supplemented as above. The third-stage larvae were washed three times and cultured in 5 ml of the medium supplemented with 10% fetal calf serum. Forty to fifty third-stage larvae were placed in a 50 ml canted neck tissue culture flask (Costar, USA) containing LLC-MK<sub>2</sub> rhesus monkey cell line as a feeder layer ( $5 \times 10^5$  cells per flask). The parasite was incubated at 37°C in 5% CO<sub>2</sub>. The media was collected and replaced every alternate day until day 18 when the worms were dead. The culture fluid was centrifuged at 3,000 rpm, 4°C for 10 min and the supernatant obtained was stored at -20°C.

#### Vaccination of jirds with irradiated filarial infective larvae

Jirds were vaccinated with irradiated infective third-stage larvae of *B. pahangi* by methods described by Chusattayanond and Denham, 1983. Batches of approximately 700 L<sub>3</sub> which have been washed three times with medium 199 (Wellcome Foundation) were suspended in 7.5 ml of fresh 199 in 20 ml scintillation vials and irradiated at 25 and 45 krad from a <sup>60</sup>Co source (Office of Atomic Energy for Peace, Bangkok). Groups of 20 jirds were vaccinated infected with 3 doses of 50 L<sub>3</sub> that have been irradiated at 25 or 45 krad at 2-week intervals.

### Antisera

Naturally infected persons and cats and experimentally infected cats and jirds were bled. Antisera were obtained from clot blood, centrifuged at 1,800 rpm for 15 min and stored at -20°C.

Human antisera Five ml of venous blood was taken from patients suffering from symptomatic or microfilaraemic filariasis in endemic areas of Thailand by the Filariasis Division, Department of Communicable Diseases Control, Ministry of Public Health, Bangkok.

Cat antisera Approximately 3 ml of blood was taken from femoral vein of *B. pahangi* and *B. malayi* infected cats.

Jird antisera Antisera were obtained from blood taken by cardiac puncture of *Brugia* infected jirds shortly after being killed by deep ether anaesthetization. The animals were infected for at least 2 months with L<sub>3</sub>s of *Brugia pahangi* or *B. malayi* by either subcutaneous or intraperitoneal route. Microfilariae were found in blood of those animals that had been infected subcutaneously and were found in the peritoneal cavity of the animals that had been infected intraperitoneally. Antisera were also obtained by the same technique from jirds that have been vaccinated with irradiated L<sub>3</sub>s of *B. pahangi* 10 days after the last dose of vaccinating infection.

## Work done to date

### I. Expansion of experimental model

The existing full life cycle animal model of E. pahangi and B. malayi have been expanded such that sufficient quantity of antigens of each of all parasitic stages can be generated for the studies. Eighteen cats and over two hundred jirds are available for laboratory use. Reproductive infections of E. pahangi and B. malayi are maintained in 8 and 6 cats respectively. These animals provide the source of infection to our established colony of the mosquitoes thus enable us to produce a large batch of infective third-stage larvae. In addition, 3 cats naturally infected with B. malayi and 1 cat naturally infected with Dirofilaria repens brought recently from the endemic area of B. malayi in Southern Thailand are also kept in our cat unit.

Intraperitoneally infected jirds with E. pahangi and B. malayi are also available as the source of adult worms and microfilariae.

### II. Collection of worms and antigen preparations

#### Filarial worms and antigens

Excretory-secretory and moulting antigens of infective third-stage larvae of E. pahangi have been prepared. Several developmental stage of filarial worms have been collected and kept frozen in small amount of saline. As summarized in Table 1, we have collected infective third-stage larvae, adults and microfilariae of E. pahangi and B. malayi as well as adults and microfilariae of D. immitis. They were all stored at -20°C.

#### Non-filarial worms and antigens

Infective third-stage larvae of A. cantonensis and G. spinigium and adult worms of T. canis have been collected for somatic antigen preparations as listed in Table 2.

### III. Collection of antisera

As mentioned in the Materials and Methods, antisera against filarial worms have been collected from human and experimental animals. All types of antisera that have been collected are listed in Table 3.

Table 1 Developmental stages of filarial worms that have been collected for somatic antigens and the ES-moulting antigens that have been prepared.

Parasites	Developmental stages collected			ES-moulting antigens of infective L <sub>3</sub>
	L <sub>3</sub>	Adult	mf	
<u>B. pahangi</u>	+	+	+	+
<u>B. malayi</u>	+	+	+	-
<u>D. immitis</u>	-	+	+	-

Table 2 Non-filarial worms that have been collected for somatic antigen preparations

Parasites	Developmental stages collected	
	L <sub>3</sub>	Adult
<u>A. cantonensis</u>	+	-
<u>G. spinigerum</u>	+	-
<u>T. canis</u>	-	+

Table 3 Antisera against Brugia parasites that have been collected from human, cat and jird

Host	Parasite	Type of infection	Route of infection	Stage of infection
Man	<u>B. malayi</u>	Natural		1. Amicrofilaraemic (Symptomatic)
				2. Microfilaraemic
	<u>W. bancrofti</u>	Natural		1. Amicrofilaraemic (Symptomatic)
				2. Microfilaraemic
Cat	<u>B. pahangi</u>	Experimental	Subcutaneous	1. Microfilaraemic
				2. Amicrofilaraemic
	<u>B. malayi</u>	Natural		Microfilaraemic
Jird	<u>B. pahangi</u>	Experimental	Subcutaneous	Microfilaraemic
			Intraperitoneal	-
		Experimental (vaccination infection with irradiated L <sub>3</sub> )	Subcutaneous	Abortive infection
	<u>B. malayi</u>	Experimental	Intraperitoneal	-

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### Workplan for the next period

In the next period we plan to

1. visit our project consultant, Dr. D.A. Denham, in London to discuss the way in which the project is progressing as well as visiting laboratories at which important work on filariasis is carrying on. Some other filarial antigens not available in our laboratory will be obtained from Dr. Denham and his colleagues.

2. collect infective third stage larvae of D. immitis from our colony of mosquitoes which will be allowed to feed on either anaesthetized microfilaraemic dogs or dog's blood containing microfilariae using membrane feeding technique.

3. collect infective larvae of Necator americanus for antigen preparations by culturing eggs obtained from human faeces.

4. collect anti-coagulated blood of people actively infected with W. bancrofti. Microfilariae will be separated from the blood. Some blood will be membrane fed to mosquitoes for infective third stage collection. These two stages of the parasite will be used for antigen preparations.

5. raise polyclonal antibodies in rabbits and/or mice against each of the antigenic preparations of filarial and non-filarial parasites.

6. start producing monoclonal antibodies against antigens from adult stage of B. pahangi and B. malayi

7. investigate the cuticle of microfilaria and adult parasites of B. pahangi and B. malayi by light, transmission and scanning electron microscopies in order to understand the basic structure of this important potential source of secretory antigens. Then polyclonal antibodies from antisera of infected humans, cats and jirds will be used to detect the sources of natural antigens in parasites' sections; substantial

portion of circulating antigens are expected to be released from the surface of cuticle. The sites of labelling with natural antibodies will be detected by immunofluorescence technique, using secondary antibodies against cat and jird IgG, labelled with fluorescein isothiocyanate, as probe. Alternatively, the sites of binding of primary antibodies will also be detected by using biotinylated protein A- Avidin D- biotinylated horseradish peroxidase system as probe. The latter technique will be performed at both light and electron microscopic levels.